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A Practical Guide to the Developmental Biology of Terrestrial-Breeding Frogs

RICHARD P. ELINSON[1], EUGENIA M. DEL PINO[2], DANIEL S. TOWNSEND[3], FABIÁN C. CUESTA[2], AND PETER EICHHORN[4]

[1]*Department of Zoology, University of Toronto, Toronto M5S 1A1, Canada,* [2]*Pontificia Universidad Católica del Ecuador, Departamento de Ciencias Biológicas, Quito, Ecuador,* [3]*Department of Biology, University of Scranton, Scranton, Pennsylvania 18510, and* [4]*Institute for Cell and Tumor Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg 1, Federal Republic of Germany*

Abstract. Many frogs lay their eggs in water; the development of these frogs is well-known. However, many frogs reproduce on land; their eggs are large and have an altered early development. As examples, *Gastrotheca riobambae* broods its embryos in a pouch on the mother's back, and *Eleutherodactylus coqui* exhibits direct development with no tadpole stage. We provide practical information on obtaining eggs and embryos from these terrestrial-breeding species and on analyzing their development. Our aim is to make these species more accessible to researchers who are interested in the developmental and evolutionary consequences of terrestrial development.

Introduction

Our view of frog development is colored by the fact that most scientists work in the temperate climates of Europe, Asia, and North America. We expect frogs and other anuran amphibians to lay their eggs in water, to develop first into tadpoles, and to metamorphose later into adults. A number of anurans, particularly those found in the tropics, do not follow this life history (Lamotte and Lescure, 1977; Duellman and Trueb, 1986). Some anurans lack a free-living tadpole and develop directly to an adult morphology. Many anurans develop entirely on land and brood their embryos in such diverse places as the oviduct, a back pouch, the stomach, or the male's vocal sac. Given these terrestrial life histories, the name "amphibian" might not have been applied to this class had taxonomy begun as a tropical science, and our view of frog development might be totally different.

Anurans with terrestrial development generally have a small number (1–150) of very large eggs (3–10 mm), which differ in various aspects of development from typical anurans. Developmental biologists have noticed only a few of these animals including *Gastrotheca riobambae, Flectonotus pygmaeus,* and *Eleutherodactylus coqui. Gastrotheca riobambae* is an egg-brooding frog from Ecuador. The female incubates the eggs in a pouch on her back, and the young are born as advanced tadpoles. *Flectonotus pygmaeus,* from Venezuela, exhibits multinucleate oogenesis. The early oocytes have up to 2000 nuclei, of which all but one disappear (del Pino and Humphries, 1978). *Eleutherodactylus coqui,* from Puerto Rico, exhibits direct development. The large eggs are brooded on land by the male (Townsend *et al.,* 1984), and the froglets hatch after about three weeks.

Developmental studies to date have concentrated on oogenesis and early development in *G. riobambae* and *F. pygmaeus* (del Pino and Humphries, 1978; del Pino and Escobar, 1981; Elinson and del Pino, 1985; del Pino *et al.,* 1986; del Pino, 1989) and on organ formation and direct development in *E. coqui* (Lynn, 1942; Lynn and Peadon, 1955; Adamson *et al.,* 1960; Chibon, 1962; Elinson, 1990). Our discussion of these animals will reflect this bias, as we describe how to obtain and work with adults and embryos of *G. riobambae, F. pygmaeus,* and *E. coqui.*

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Gastrotheca riobambae and Other Egg-Brooding Frogs (Hylidae)

Collection and maintenance[1]

Sites of collection. The majority of egg-brooding frogs inhabit the humid forests of northern South America, have very limited distribution ranges, and are rarely found (Table I). Species of *Gastrotheca* that inhabit the highlands of the Andes, however, occur in large numbers and are more easily collected. We will mostly discuss *G. riobambae,* the species with which we have extensive experience, and will make reference to *G. plumbea* and *F. pygmaeus,* which we have also maintained in captivity.

Gastrotheca riobambae occurs in the interandean valleys of northern Ecuador at altitudes of 2500–3200 m (Duellman and Hillis, 1987). Frogs are found along the banks of irrigation ditches and under stones in humid areas, as well as sitting on vegetation. This frog gives birth to tadpoles that develop in temporary pools and lakes. The free-living tadpole stage lasts several months. The large tadpoles of *G. riobambae* (around 70 mm total length) are the only amphibian larvae that exploit the standing bodies of water in the highlands of northern Ecuador.

Gastrotheca plumbea lives in cloud forests at elevations of 1300–2350 m on the Pacific slopes of the Andes in Ecuador (Duellman and Hillis, 1987). Both adults and young are found in axils of large bromeliads. *Gastrotheca plumbea* exhibits direct development, which means it lacks a free-living tadpole stage.

Flectonotus pygmaeus lives in axils of bromeliads, in the cloud forest at Estación Biológica de Rancho Grande, Maracay, Venezuela. This frog gives birth to advanced, non-feeding tadpoles that complete metamorphosis in about a week. The tadpoles are deposited in the water collected in axils of bromeliads (Duellman and Maness, 1980).

Table I

Geographic distribution of egg-brooding frogs

Genera of egg-brooding frogs	Approximate number of species[a]	Distribution[a]
	WITHOUT POUCH	
Cryptobatrachus	3	Andes and Sierra Nevada de Santa Marta, Colombia
Hemiphractus	5	Panama, Pacific slopes of Colombia and northwestern Ecuador; upper Amazon Basin in Brazil, Ecuador, Perú and Bolivia[b]
Stefania	7	Highlands of the Guyana Shield in Venezuela and Guyana[c]
	MARSUPIAL FROGS	
Flectonotus	2	Coastal Cordillera of northern Venezuela, Tobago and Trinidad[d]
Fritziana	3	Mountains of Southeastern Brazil (Guanabara, Minas Gerais, Rio de Janeiro, Sao Paulo)[d]
Gastrotheca	41	Panama, northern and western South America, southward to northern Argentina, east and southeastern Brazil

[a] Duellman (1977).
[b] Trueb (1974).
[c] Duellman and Hoogmoed (1984).
[d] Duellman and Gray (1983).

Terraria for laboratory maintenance. Our terraria for maintaining *Gastrotheca* and *Flectonotus* consist of wooden frames, 60–80 cm in length by 40 cm in width and 40–50 cm in height, with side walls of plastic mesh. The floor and the roof are made of wood. The roof has a large door, that fits tightly, and measures about half the length of the terrarium. Terraria of glass and of plastic are also used. In those cases, the cover is made of plastic or metal mesh to allow gas exchange.

The number of frogs per terrarium varies according to frog size. We keep about 8–10 adults of *G. riobambae,* 4–5 adults of the larger *G. plumbea,* or 12 *F. pygmaeus* per terrarium. *Gastrotheca riobambae* have been kept for up to five years, with reproduction occurring about once a year. *Gastrotheca plumbea* and *F. pygmaeus* have been kept for about two years, but their reproductive cycle and lifespan are not known.

Inside terraria we place large plastic trays with earth to cover the floor completely. Stones and hollow pieces of

[1] South American wildlife is protected by law. Investigators interested in the collection of these frogs should check with the appropriate authorities in the country where they wish to work. Permission for the collection and export of frogs from Ecuador is given by the Director, Dirección de Desarrollo Forestal, Ministerio de Agricultura, Quito, Ecuador. A proposal, written in Spanish, should be sent several months in advance to the above address with a copy to the collaborating investigator or institution in Ecuador. It should be accompanied by the curriculum vitae of the investigator and a letter of support from the researcher's home institution. Duplicate collections must remain in Ecuador, usually at the Museo de Ciencias Naturales, Casa de la Cultura Ecuatoriana, Quito, Ecuador, or at the collection of one of the universities. To obtain permits, visiting scientists are required to give lectures about their work at the Museo de Ciencias Naturales or at the collaborating institution. Upon completion of the work, a report and copies of published works should be sent to Dirección de Desarrollo Forestal and to the collaborating institutions. Upon arrival in Quito, investigators are advised to visit the Dirección de Desarrollo Forestal as soon as possible to get their permits.

Investigators interested in *F. pygmaeus* are advised to check with the Estación Biológica de Rancho Grande, Maracay, Venezuela. A permit for collection is required from the Estación Biológica de Rancho Grande, and permits for export are obtained from the Oficina de Fauna, Ministerio de Agricultura y Cria, Caracas, Venezuela.

brick provide frogs with hiding places, and branches serve as perches. One or two small containers with water (about 5 cm in depth) are provided with stones to give support to the frogs. Each terrarium contains one or two small pots planted with *Tradescantia*. This plant grows easily and soon covers the terrarium. In addition, a bromeliad is planted, when available. Bromeliads with thorny leaves produce wounds in frogs and are not recommended. Terraria are placed near windows where there is ample sunlight and are kept moist by watering at least twice a week. Terraria are kept at 17–23°C. In native habitats of *G. riobambae*, the temperature fluctuates between 5°C at night to 23°C at midday.

Favorite hiding places for both *G. riobambae* and *G. plumbea* are the cavities under stones and bricks and in axils of bromeliads. In addition, they hide in the crevices between plastic trays and the terrarium walls as well as under the vegetation. *Flectonotus pygmaeus* perches in axils of bromeliads and on the vegetation. Frogs bask in the sun and may remain in the same place for several days. Frogs are in the water often, so water must be changed frequently.

We have not tried terraria of larger height. However, Zimmermann (1983) described taller terraria with several vegetation levels and high humidity for the maintenance of tropical frogs. Those terraria have a frontal glass door and might be useful for maintaining large arboreal species of egg-brooding frogs.

Feeding. Adult *G. riobambae* are fed two to three times a week. The easiest, most efficient food consists of sowbugs (*Porcellio* sp.) or meal-worm larvae (*Tenebrio mollitor*), mixed with small pieces of dry dog food. Food is placed in shallow plastic containers (1.5–2 cm in depth) and is always given in the same place. Meal worms remain in feeding containers and in this way, frogs learn to eat dry food when capturing prey.

We have maintained *G. riobambae* successfully on only a sowbug diet; however, sowbugs become scarce in dry weather. To avoid food scarcity, feeding alternately with sowbugs or meal-worms and dry dog food gives excellent results. Dog food provides the frogs with carbohydrates, lipids, vitamins, and minerals. Outside the feeding containers, frogs ingest dirt from the terrarium floor with their prey and may obtain needed trace elements; frogs raised without dirt become weak. This diet is also well accepted by *G. plumbea*. *Flectonotus pygmaeus* is fed once or twice a day on large *Drosophila* caught from the wild. Small sowbugs and meal-worm larvae were not tried, but could probably be given successfully to these frogs.

Newly metamorphosed *G. riobambae* readily accept small meal-worm larvae, and this type of food is a key to successfully raising these frogs when used in combination with other small prey items. We mix larvae with dry dog food in very shallow plastic containers. Newly born *G. plumbea* have been raised successfully on the same diet given to young *G. riobambae*. In addition, young *G. plumbea* were fed large *Drosophila* caught from the wild. Juvenile *G. riobambae* and *G. plumbea* reach the adult stage 8–12 months after metamorphosis. Newly metamorphosed *F. pygmaeus* are quite small, and we have had no success in raising them. Very small meal-worm larvae, which are readily accepted by other species, were not tried on *F. pygmaeus*.

Amplexus and birth. Before amplexus, *G. riobambae* males call frequently. Amplexus occurs on land and it lasts for 24–48 h before egg-laying begins. Egg laying lasts about 6–8 h. During egg laying, the male introduces his feet inside the female's pouch. As each egg leaves the female's cloaca, the male catches it with his heels and toes and moves it inside the pouch, the opening of which is about a centimeter anterior. In this way, eggs do not touch the ground. Eggs leave the female's cloaca, one at a time, at intervals of 30–60 s. A few eggs are lost and remain in the soil at the end of amplexus. Fertilization probably occurs during the egg's journey from the female's cloaca to the pouch.

After amplexus, the female places herself tight against the cavity of a stone or other object, probably to help in pouch distension and in the arrangement of embryos in one or two even layers (del Pino *et al.*, 1975). Incubation of embryos lasts a mean of 100 days, during which time the wet weight of embryos increases three-fold, while the dry weight remains constant (del Pino and Escobar, 1981). At birth, the total weight of embryos equals one third to one half the weight of the female. Females become so swollen with embryos that their movements are greatly reduced.

At birth, the female enters the water, introduces the long toes of her hind legs inside the pouch, and aids in the removal of tadpoles. She supports herself with her front legs against the walls of the water container or against a stone. Tadpoles at birth measure 18–20 mm in total length (del Pino *et al.*, 1975).

In captivity, amplexus of *G. riobambae* occurs between September and February, the period with heavier rainfall in Quito. Sometimes frogs mate in June. These periods seem to coincide with times when most reproduction occurs in nature; however, females with embryos can be collected throughout the year. By administering human Chorionic Gonadotropin (hCG) to male and female frogs, described later, amplexus and reproduction can be obtained in captivity at any time of the year.

Amplexus in *G. plumbea* also occurs on land, but details have not been documented. Incubation lasts about 120 days (del Pino and Escobar, 1981). At birth, the mother actively aids in the elimination of offspring by stretching the opening of the pouch and digging young out of the pouch with her hind feet, as in *G. riobambae* (Duellman

and Maness, 1980). Brooding females have been found in June and July, and frogs in captivity gave birth in September (Duellman and Maness, 1980). Amplexus and birth for *F. pygmaeus* have been described by Duellman and Maness (1980). Amplexus occurs on land, and at birth the female deposits larvae in water. Embryos were incubated for 29 days in captivity (del Pino and Escobar, 1981). The breeding season of *F. pygmaeus* at Estación Biológica de Rancho Grande spans from April until early November, the time of the year with the heaviest rainfall (Duellman and Maness, 1980). We have not tried hormonal stimulation of reproduction in *G. plumbea* or *F. pygmaeus*, but both species have mated spontaneously in the laboratory.

Care of tadpoles. The extensive use of pesticides and urban growth have diminished the populations of *G. riobambae*, so frogs must be collected from remote localities. To ensure a supply of frogs, we have developed methods for raising frogs from tadpoles.

Tadpoles are best maintained in large tanks with at least one cubic meter of water, a depth of 30–50 cm, and a temperature of 17–21°C, without changes of water. Tanks are provided with stones as hiding places, a large plant (*Cyperaceae*) whose roots, floating stems, and leaves provided places for tadpoles to sit near the water surface, and a few branches of *Elodea*. These tanks support an abundant population of algae and protozoans that become the main constituent of the tadpole diet. About 70–100 tadpoles are raised per tank. Little or no cannibalism is observed, and tadpoles grow normally, even when newly born tadpoles are placed with older ones.

Tadpoles of *G. riobambae* are voracious eaters. The easiest, most effective diet has been small pieces of dry dog food or rabbit food given once or twice a week, supplemented by the algal growth in the tanks. Care should be taken not to contaminate the water with too much food. Tadpoles reached 70 mm total length before metamorphosis, at about 3 months after birth. Frogs measured 18–25 mm snout-vent length, looked healthy, and survived after metamorphosis.

Tadpoles have also been kept in small glass and plastic aquaria of 10–20 liters capacity. Without a stable population of algae, the water becomes contaminated and requires weekly changes. In some instances, aquaria were aerated by means of an aquarium pump, but we found no advantage in aeration. Tadpoles of *G. riobambae* have well-developed lungs by the time of birth and take oxygen from air. Population densities in aquaria correspond to 1–3 tadpoles per liter of water. Higher densities result in cannibalism and early metamorphosis. Under crowded conditions small tadpoles, 50–60 mm total length, reached metamorphosis in only 40 days at 18°C. The newly metamorphosed frogs were small (10–15 mm snout-vent length), extremely weak, and often died at metamorphosis or soon thereafter.

We have tested a variety of other diets for tadpoles. Diets included cooked and raw meat; cooked lettuce, chard and spinach; chicken feed hardened with agar; egg yolk from cooked eggs, and fish food for aquaria (Tetramin, Tetra Werke, 4520 Melle, Federal Republic of Germany). Of these diets, fish food is the best, followed by chicken feed hardened with agar.

Flectonotus pygmaeus produces about six advanced tadpoles per breeding season. In the laboratory, tadpoles have been kept in shallow water containers. These tadpoles do not eat and only need 2–4 weeks of aquatic living to reach metamorphosis (Duellman and Maness, 1980).

Maintenance at lower altitudes. The habitat of *G. riobambae* is the high montane environments in northern Ecuador (2500–3200 m altitude). Atmospheric pressure, oxygen availability, length of daylight, and amount of sunlight as well as temperature of the *G. riobambae* habitat differ from the conditions of most laboratories where studies on development are conducted. Change in altitude and temperature often result in frog death. We had the experience of raising *G. riobambae* at the aquaria of the German Cancer Research Center in Heidelberg, Federal Republic of Germany. Most female frogs that were incubating embryos died a few days after arrival, but one frog gave birth successfully and provided us with tadpoles, from which we obtained a supply of frogs.

Tadpoles were raised in small glass aquaria at a density of three tadpoles per liter. Aeration was provided with aquarium pumps. Tadpoles were fed fish food (Tetramin) and cooked lettuce and attained the normal length of about 70 mm before metamorphosis, about one month after birth. Newly metamorphosed frogs ate flies and small crickets readily. Eight months after birth, frogs reached adult size and began to sing. Adult frogs were maintained in terraria as previously described. Temperature was adjusted to 17–18°C and the light regime was a 12-h light/dark cycle. Frogs were fed adult house-flies and medium-size crickets, which were often dusted in powdered vitamins and minerals, Osspulvit (Zimmermann, 1983), to supplement the diet. Amplexus was observed on several occasions; however, egg laying did not occur.

Accelerated development has been reported previously at lower altitude. In Holland, Hoogmoed (1967) obtained metamorphosis of *G. riobambae* tadpoles in 41 days after birth at a temperature of 21–26°C. In contrast, tadpoles normally require several months of aquatic living to reach metamorphosis in Quito. Water temperature as well as the change in altitude should be factors involved in the differences observed.

We induced amplexus and reproduction by administering hCG intraperitoneally to both male and female frogs, as described later. Amplexus and fertilization oc-

curred normally. Eggs began to cleave, but died at around the time of gastrulation. Lower altitude seems to affect the physiological condition of frogs and may affect the quality of eggs. For instance, the induction of oocyte maturation by progesterone *in vitro*, took 10 h longer in Santiago de Chile than in Quito (de Albuja *et al.*, 1983). Auber-Thomay and Letellier (1986) obtained regular and spontaneous reproduction of *G. riobambae* in France. Incubation took 41–74 days, but half of the eggs from 10 frogs failed to develop. In contrast, 75–108 days of incubation are needed in Quito, but almost all of the eggs (99.4%) developed (del Pino and Escobar, 1981). Our experience, and that of others, indicate that successful reproduction, as it happens in Quito, is rare at lower altitudes.

Oogenesis, fertilization, and the culture of embryos

Analysis of oogenesis. Egg-brooding frogs have synchronous oogenesis, which means that only one batch of oocytes grows in the ovary at one time (del Pino *et al.*, 1986). Full-grown oocytes are the largest documented among anurans, reaching 2.5–10 mm in diameter, depending on the species (Table II). Their volume is primarily due to yolk, and oocytes of *G. riobambae* actually have less rRNA than oocytes of *Xenopus laevis*, which are $^1/_{16}$th the volume (del Pino *et al.*, 1986).

In some species of egg-brooding frogs, oocytes contain many nuclei (4–2000 nuclei depending on species) during the previtellogenic period (Table II; del Pino and Humphries, 1978). At vitellogenesis, only one nucleus remains as the oocyte's germinal vesicle while the rest degenerate. This type of oogenesis is called multinucleate oogenesis (del Pino and Humphries, 1978), and unfortunately, the analysis of it is limited by the availability of frogs. Marsupial frogs with multinucleate oocytes are mostly large frogs (Table II) that live in cloud and humid forests and are rarely found. The most accessible species encountered so far is *F. pygmaeus*.

To study oogenesis, ovarian pieces are removed from the body cavity of the frog or tadpole and are placed in modified Barth Solution (MBS) (Gurdon, 1968), amphibian Ringer's, or in other amphibian saline solutions. MBS contains 88 m*M* NaCl, 1 m*M* KCl, 2.4 m*M* $NaHCO_3$, 0.82 m*M* $MgSO_4$, 0.33 m*M* $Ca(NO_3)_2$, 0.41 m*M* $CaCl_2$, 10 mg/l each benzylpenicillin and streptomycin sulphate and 2 m*M* Tris-HCl (or 10 m*M* Hepes), pH 7.6.

Methods for the cytological observation of germinal vesicles in mono- or multinucleate oocytes of egg-brooding frogs do not differ from those used with other amphibia (Macgregor and del Pino, 1982). The large amount of yolk stored in large oocytes, however, is bothersome for cytology as well as for the extraction of nucleic acids.

Fixation of oocytes for cytological studies needs to be modified due to the yolk content of oocytes. It is important to increase the volume of fixative and the length of fixation times. Smith's fluid (Rugh, 1965) is our fixative of choice for paraffin and plastic sections. One or two yolky oocytes of *G. riobambae* are fixed in 10–20 ml of Smith's fluid for 12–24 h in the dark, washed in distilled water with several changes for 24 h, and stored in 4% neutralized formalin until processing. Yolky oocytes fixed in Smith's are particularly easy to section, when embedded in paraffin. Material embedded in plastic resins, like JB-4 (Polysciences), provide better resolution than paraffin sections.

Small oocytes, taken from large tadpoles and newly metamorphosed frogs, can be processed for electron microscopy according to standard methods. Large, yolky oocytes should be cut into smaller pieces during glutaraldehyde fixation to allow better penetration of fixative. Thick epon sections (0.5–1 μm thickness) of oocytes, fixed in glutaraldehyde and postfixed in osmium tetroxide, can be used for light microscopy. Sections are placed on a drop of water over a clean slide and, after drying, are mounted with immersion oil for light microscopy (del Pino *et al.*, 1986).

The study of nucleic acids in large oocytes of *G. riobambae* is affected by the amount of yolk. The volume of working solutions needs to be increased 10 times in comparison to *X. laevis* oocytes due to the large oocyte volume. Nucleic acids cannot be totally cleaned from yolk contaminants without losing small RNA molecules (del Pino *et al.*, 1986).

The large size of oocytes in egg-brooding frogs is, at first, attractive for microinjection experiments. There are, however, several limitations. The small number of eggs and oocytes per female (Table II) prevent their extensive use. Eggs are uniformly pale, and the animal pole cannot be easily distinguished, a limitation when microinjection into the germinal vesicle is desired. Large egg size is due to yolk content, and the germinal vesicle of *G. riobambae* oocytes is equivalent in size to the germinal vesicle of *X. laevis* oocytes (del Pino *et al.*, 1986). Microinjection into oocytes and eggs of *G. riobambae* is further complicated by their high internal pressure. Oocytes and eggs often burst after microinjection, although this can be prevented by injecting oocytes kept in a humid chamber or in 5% Ficoll in an amphibian saline. The easily available oocytes of *X. laevis* provide superior material for microinjection experiments.

Hormonal induction of oocyte maturation, ovulation, and mating in G. riobambae. Large oocytes within the ovarian follicle can be induced to undergo germinal vesicle breakdown (GVBD) *in vitro* by exposure to hCG or progesterone. In contrast, oocytes denuded from the follicular wall undergo GVBD only in response to progesterone. Treatment of ovarian follicles with hCG results not only

Table II

Oogenesis and development of egg-brooding frogs

Frog species[a]	Snout-vent length of female (mm)	Number of oocyte nuclei 1 = mononucleate 2 = multinucleate	Egg diameter (mm)	Clutch size	Development 1 = tadpole 2 = direct
		FROGS WITHOUT POUCH			
Cryptobatrachus boulengeri	65	—	4	26	2
C. fuhrmanni	60	1	4	28	2
Hemiphractus bubalus	58[c]	1	—	—	2
H. fasciatus	57[c]	1	7	13	2
H. johnsoni	68[c]	2	9	18	2
H. proboscideus	56[c]	1	—	—	2
H. scutatus	70[c]	1	10	10	2
Stefania evansi	64[d]	2	9[d]	11[d]	2
S. ginesi	61[d]	—	9[d]	8[d]	2
S. goini	93[d]	—	9[d]	15	2
S. woodleyi	61[d]	—	9[d]	6[d]	—
		MARSUPIAL FROGS			
Frogs with pouch type one[b]					
Flectonotus fitzgeraldi	19[e]	2	3	3	2
F. pygmaeus	26[e]	2	3	7	2
Fritziana fissilis	26[e]	1	3	10[e]	1
F. goeldii	32[e]	1	4	15[e]	1
F. ohausi	25[e]	1	—	—	1
Frogs with pouch type two[b]					
Gastrotheca guentheri	82	2	—	—	2
G. andaquiensis	74	—	9	10	2
G. cornuta	74	2	10	10	2
G. dendronastes[f]	69	2	8	12	2
G. longipes	95	—	8	17	2
G. ovifera	81	2	8	32	2
G. microdisca[g]	75[g]	2	8	23[g]	2
G. walkeri[h]	60	2	7	19[h]	2
G. weinlandii	85	2	10	14	2
Frogs with pouch types three and four[h]					
G. christiani[i]	36	1	4	10	2
G. excubitor	41	1	6	10	2
G. galeata[j]	52[j]	1	5	20	2
G. griswoldi	41	2	8	12	2
G. ochoai	38	1	5	—	2
G. testudinea	60	1	5	30	2
Frogs with pouch types five and six[b]					
G. argenteovirens	42	1	3	35	1
G. aureomaculata	75	—	5	70	1
G. fissipes	81[g]	2	10[g]	24	2
G. gracilis	40	1	4	68[j]	1
G. lojana	60	1	3	—	1
G. nicefori	73	—	8	30	2
G. peruana	53	1	3	—	1
G. plumbea	69	1	5	28	2
G. orophylax[k]	59[k]	1	6[k]	21[k]	2
G. marsupiata	42	1	2.5	138	1
G. monticola	59[l]	1	3	80	1
G. riobambae	49[l]	1	3	128	1

[a] Duellman (1977).
[b] del Pino (1980).
[c] Trueb (1974).
[d] Duellman and Hoogmoed (1984).
[e] Duellman and Gray (1983).
[f] Duellman (1983).
[g] Duellman (1984).
[h] Duellman (1980).
[i] Laurent et al. (1986).
[j] Trueb and Duellman (1978).
[k] Duellman and Pyles (1980).
[l] Duellman and Hillis (1987).

in GVBD but also in ovulation (de Albuja *et al.*, 1983). The responses of *G. riobambae* oocytes to these hormones are equivalent to *X. laevis* and suggest that there are similar mechanisms for the conversion of an oocyte into an egg. The time of the response is longer in *G. riobambae*. The large oocytes of *G. riobambae* need an exposure of 15–24 h to progesterone to undergo GVBD compared to 6–8 h for *X. laevis* oocytes (de Albuja *et al.*, 1983).

To induce ovulation, female *G. riobambae* are injected intraperitoneally with hCG. The amount given ranges from 200 to 1000 IU of hCG, administered in one or two doses at 6–24 h intervals. Ovulation and egg release occurred 29–76 h after hormone injection (de Albuja *et al.*, 1983). When a female injected with hCG is placed in a terrarium with male frogs, amplexus and mating occur readily. In some instances, the male was simultaneously stimulated by the intraperitoneal injection of 200–500 IU of hCG. Hormonal stimulation of amplexus and mating in *G. riobambae* greatly facilitates the study of reproduction in this frog.

The maternal pouch as an indicator of the female's reproductive condition. The pouch of *G. riobambae* is a sac of integument located on the back of the female, under the dorsal skin. The pouch is essentially independent from the skin, except at the aperture, where it is continuous with the dorsal skin. Pouch integument of frogs that are not incubating embryos is similar to frog skin. It differs from skin by the presence of abundant mucous glands, fewer serous glands, and the lack of keratinization of the epithelial layer (Jones *et al.*, 1973; del Pino *et al.*, 1975).

The pouch of *G. riobambae* opens in the midline of the female's back and the aperture has an inverted V or U shape. The pouch can be open or closed according to the reproductive condition of the female (Fig. 1). The pouch of females with small ovaries is open; it closes when ovaries are large and the female is ready for reproduction, and it remains closed during the period of embryonic incubation. The pouch opens again at birth (del Pino, 1983). The condition of the pouch aperture provides an important parameter for the selection of female frogs that can reproduce readily. The dose of hCG needed to stimulate amplexus and mating in frogs with a closed pouch is smaller than that in frogs with open pouches. Similarly, oocytes of frogs with closed pouches require shorter periods of exposure to progesterone to undergo GVBD (de Albuja *et al.*, 1983; del Pino, 1983).

Incubation of embryos results in great distension and attenuation of the pouch in *G. riobambae*. The pouch, which originally occupied a small portion of the female's back, increases in size during incubation until it occupies the entire back and sides of the body. During the first weeks of incubation, the walls of the pouch become highly vascularized and grow between embryos, forming partitions that tightly envelop each embryo in a vascularized

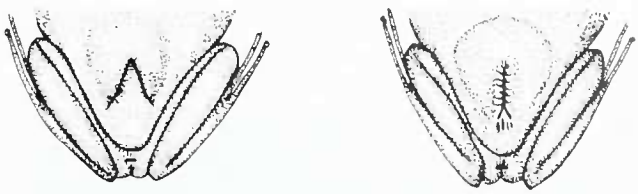

Figure 1. The pouch on the back of a *Gastrotheca riobambae* female. a. When the female is not incubating embryos, the pouch has an inverted V-shaped opening with smooth borders. b. When the female is ready to ovulate or is incubating embryos, the pouch closes with the borders touching except at the posterior end. (From del Pino, 1983.)

chamber. Embryos, in turn, develop disk-shaped gills, the bell gills (Noble, 1927), that envelop the growing embryo in a vascularized gill sac.

Fertilization. Fertilization in *G. riobambae*, *G. plumbea*, and *F. pygmaeus* is external, but it does not occur in water as in most frogs. Eggs are fertilized during the journey from the female's cloaca to the pouch, as already discussed. Since the requirements for *in vitro* fertilization are unknown, we depend on normal mating of frogs to obtain fertilized eggs.

When testis of *G. riobambae* are macerated in full strength MBS, sperm cells show little motility, but remain alive. Saline solutions of low ionic strength (like 10% MBS) do not trigger sperm motility. Such media, in fact, result in rapid swelling and bursting of sperm. *Xenopus laevis* sperm, in contrast, swim actively in solutions of low ionic strength before dying (Wolf and Hedrick, 1971). The experience of mixing both gametes for *in vitro* fertilization has, so far, been unsuccessful. The length of time needed for the process and the possible requirement of pouch secretions to activate sperm are factors that need to be considered for an *in vitro* fertilization method.

Culture of embryos. Embryos of *G. riobambae* can be removed from the pouch without affecting the mother or the development of other embryos in the pouch. Embryos are removed with a wide probe or a blunt pair of forceps. With frequent removal of embryos and handling of the frog, some embryos die and desiccate in the pouch. Dead embryos do not affect the development of the brood. Gentle handling of the frog with a piece of wet cloth helps to avoid desiccation, and many embryos survive in the pouch. In *F. pygmaeus*, the borders of the pouch are firmly sealed. Removal of embryos breaks the seal, but the remaining embryos will continue to develop.

During early stages of incubation, removal of embryos is easy. Newly fertilized eggs can be obtained by slight pressure on the female's back in *G. riobambae*. Later, with the development of embryonic chambers, pouch tissue adheres firmly to the jelly capsule of each embryo, and the capsule and bell gills can be ruptured during removal of embryos. Dipping the tip of the probe in MBS,

before insertion into the pouch, helps in the clean separation of pouch tissue from embryos.

Segmenting eggs and early embryos of *G. riobambae* die in saline solutions of low ionic strength (*e.g.*, 10% MBS), but survive in full strength MBS or other amphibian salines. Advanced embryos (del Pino/Escobar stages 20–25) survive in solutions of high and low ionic strength (del Pino *et al.*, 1975). The tolerance of advanced embryos to low salt concentration is a preparation for the free-living tadpole stages.

For *in vitro* culture, segmenting eggs and early embryos are placed in 5% Ficoll in an amphibian saline. Embryos at the onset of cleavage have been cultured for 20 days in a tissue culture well filled with the above solution. Ficoll prevents swelling, which occurs in saline solutions and which inhibits gastrulation and leads to bursting. Culturing in Ficoll permits observations of blastula and gastrula stages. Successful culture of cleavage stage embryos was also accomplished by placing embryos in MBS after removing them from the pouch and transferring them to 2-ml wells of disposable tissue culture plates with a 10 μl drop of MBS. The amount of liquid around the embryo should be very small. The well is then filled with mineral oil. Once or twice a week, embryos are washed in a large dish with MBS to retard fungal growth, although it is easy to damage embryos during manipulation. Embryos can be cultured in this way for 2 to 4 weeks. We recommend using Ficoll in amphibian saline and incubation in 1–1.5 ml without changing the medium.

Advanced embryos (del Pino/Escobar stages 20–25) hatch from the jelly capsule quite easily. For culture, embryos are placed in a petri dish with full strength MBS, 10% MBS or tap water. The jelly capsule breaks, bell gills are resorbed into the peribranchial cavity, and embryos begin to swim as tadpoles. Embryonic growth in culture always becomes accelerated compared to development in the pouch. Some embryos develop edema when cultured at low salt concentrations.

Less efficient methods for the culture of segmenting eggs and early embryos of *G. riobambae* are the culture of embryos in a petri dish filled with MBS and the culture of embryos in humid chambers. In both cases, embryos hatch from the jelly capsule in a matter of days, and they swell and die. Alterations of the developmental pattern do occur; in particular, development of blood is deficient (del Pino *et al.*, 1975). Embryos (del Pino/Escobar stages 10–25) tolerate solutions of high ionic strength like 1.5 MBS and 2.0 MBS. However, the jelly capsule bursts and embryos swell and die in about 10 days. Development is retarded under those conditions.

Normal development

Development of *G. riobambae* follows the amphibian pattern, with several striking differences. Development is very slow, requiring about 12 h for first cleavage, a week until gastrulation begins, and another week for the completion of gastrulation (Elinson and del Pino, 1985). Gastrulation results in the formation of a small group of cells, called the embryonic disc, from which the embryo's body arises (del Pino and Elinson, 1983). The heart forms anterior to the head, and large bell gills develop, which envelop the embryo completely (del Pino and Escobar, 1981). Embryos up to del Pino/Escobar stage 17 are unpigmented and translucent, so that organ formation can be easily followed.

A staging table for the period of incubation has been prepared by del Pino and Escobar (1981), with further details of cleavage and gastrulation in Elinson and del Pino (1985). Salient features will be mentioned here (Fig. 2). Cleavage results in small animal micromeres and large vegetal macromeres (stage 5), and the blastula has a translucent roof (stage 6). The blastopore of gastrulation is hard

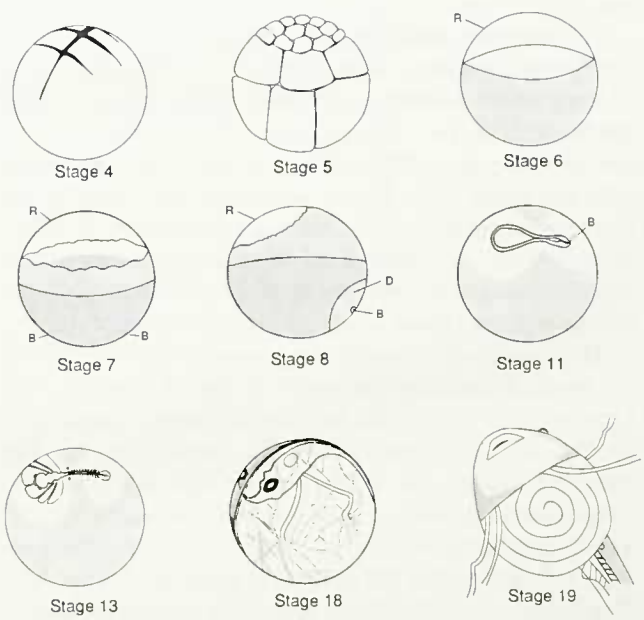

Figure 2. Selected del Pino/Escobar stages of *Gastrotheca riobambae* development. The first three cleavage furrows are vertical and cut slowly through the egg from animal to vegetal (Stage 4). The small, animal micromeres (Stage 5) become the translucent blastocoel roof (R) and the vegetal macromeres remain large (Stage 6). The faint blastoporal lip (B) forms near the vegetal pole at the start of gastrulation (Stage 7). The lip is usually impossible to see on living embryos, so the internal movement of cells along the blastocoel roof is a sign of gastrulation. The blastopore closes, and the lips become a discrete disc (D) of small cells (Stage 8). As the archenteron expands, the embryonic disc stretches to form the archenteric roof, and the whole embryo rotates with respect to gravity. Neural folds develop from the former disc cells (Stage 11). The embryo develops a central nervous system, branchial arches, and somites, all from the disc cells (Stage 13). The branchial arches produce bell gills, which expand to surround the embryo with vascular sheets (Stage 18). When the bell gills are dissected away, the characteristic tadpole is revealed (Stage 19). (Modified from del Pino and Escobar, 1981; Elinson and del Pino, 1985.)

to see because the eggs are white, but the migration of cells along the blastocoel roof is a sign of gastrulation (stage 7). The cells of the closing blastoporal lips form the embryonic disc (stage 8), which expands with the expansion of the archenteron. The embryo rotates so that the former disc is uppermost, and neural folds form from the disc cells (stage 11). The embryo develops as flat sheets of tissue with the central nervous system, somites, and branchial arches clearly visible (stage 13). The bell gills develop from the branchial arches and envelop the embryo (stage 18). With the absorption of yolk, the embryo acquires the typical tadpole appearance (stage 19). After birth, tadpoles can be staged using Gosner's (1960) generalized staging tables for aquatic species. Staging tables have not been prepared for egg-brooding frogs with direct development (Table II). These frogs also develop bell gills; there is partial development of the operculum, and precocious development of the limbs (del Pino and Escobar, 1981).

Manipulation of embryos

Few attempts have been made to manipulate embryos of *G. riobambae* for experimental purposes. Eggs of *G. riobambae* are uniformly pale, so that cleavage and gastrulation are hard to see in living embryos. By impregnating entire embryos with silver, the outline of cells can be seen (Kageyama, 1980). An embryo is removed from the pouch and its jelly coats are removed with forceps or by rolling the embryo on a piece of moist paper towel. The presence of air bubbles on the jelly should be avoided as they interfere with staining. The embryo is immersed, with agitation, in Stockerd's fixative (5 parts formalin, 4 parts acetic acid, 6 parts glycerol, 85 parts distilled water) for 10–20 s, and transferred immediately into a bath of distilled water. Longer fixation periods in Stockerd's fixative result in the rupture of cells. The egg is then placed in a dilute solution of silver nitrate prepared by adding one or two drops of a 0.5% stock solution of silver nitrate to 2 ml distilled water. Higher concentrations of silver nitrate produce dark staining of the egg. The egg is left in this solution for 1–2 min. It is washed in distilled water and exposed in distilled water, over a white background, to sunlight for a few minutes or until dark color develops. In the absence of sunlight, embryos can be exposed to the light of a strong lamp. Stained eggs are stored in 4% neutralized formalin.

Cell movement during cleavage and gastrulation can be followed by vital staining. Pieces of agar containing Nile blue sulphate are prepared by dissolving the stain in agar with boiling. The liquid is dried on a glass plate, and the resulting film is cut into small pieces. An embryo of *G. riobambae* is taken from the pouch, and its outer jelly removed by rolling the embryo on a piece of moist paper towel. The embryo is placed in a humid chamber, and spots of stain are applied to its surface using a piece of agar like a brush. After a few minutes, the embryo's surface is stained, and the excess stain, left on the jelly, is removed. Stained embryos are cultured under vaseline oil, and the changes in shape of the stain marks can be followed.

Segmenting eggs and embryos can be sectioned serially after fixation in Smith's fixative, as explained previously. Body formation and development of bell gills can be followed by means of whole mount permanent preparations. Embryos (del Pino/Escobar stages 11–17) are cut from the yolk and fixed in Bouin's solution. Embryos are then stained with borax-carmine and are mounted on a slide.

Eleutherodactylus coqui (Leptodactylidae)

Collection and maintenance[2]

Sites and methods of collection. The genus *Eleutherodactylus*, with more than 400 species, is found from Mexico to northern Argentina and southeastern Brazil, as well as the West Indies and Florida. Most species have direct development of terrestrial eggs, but *E. jasperi* is viviparous (Drewry and Jones, 1976; Wake, 1978).

Eleutherodactylus coqui is the most common of sixteen congeners native to Puerto Rico (Rivero, 1978). The species is not available through any biological supplier (currently, no *Eleutherodactylus* species is), but must be captured in its native habitat.

As implied above, *E. coqui* can be confused with several other congeners that may occur sympatrically in various parts of the island. At least two of these species have threatened or endangered status, so it is important to contact a local herpetologist who is familiar with *E. coqui*.

[2] Collection of any Puerto Rican species of frogs or eggs requires a permit. Permits can be obtained by writing to Department of Natural Resources, Apartado 5887, Puerta de Tierra, Puerto Rico 00906.

We urge any biologists interested in collecting frogs in Puerto Rico for laboratory investigations to contact a faculty member or research biologist at any of the following institutions to learn the most appropriate places for collecting without endangering local populations or disrupting ongoing field studies. Contacts include: Department of Biology, University of Puerto Rico, Rio Piedras, Puerto Rico; Department of Biology, University of Puerto Rico, Mayaguez, Puerto Rico; and Terrestrial Ecology, El Verde Field Station, GPO Box 3682, San Juan, Puerto Rico 00936.

The El Verde Field Station is located within the Luquillo Experimental Division of the Caribbean National Forest. El Verde Field Station is on the northwestern flanks of El Yunque, one of the principal peaks of the Luquillo Mountains. The Experimental Forest surrounding the station at El Verde is strictly for research on the properties and processes of the rain forest. *Eleutherodactylus coqui* is the most abundant of eight *Eleutherodactylus* species that inhabit the forest at El Verde. The station is open year-round to research biologists as space and scheduling permit. Information on facilities and research opportunities at the station may be obtained by writing to the Director of Terrestrial Ecology at the above address.

In their native habitat, "coquíes" can be collected most easily during their nocturnal activity period. Males call from fairly exposed elevated perches and are quite accessible. Females, which are silent, are more difficult to locate. Females often climb out of reach on wetter nights, so few may be found near the ground (Stewart, 1985). Daytime collecting, consisting of searching the leaf litter, is less productive in terms of adult animals, but may reward the searcher with a higher incidence of developing clutches of *E. coqui*, usually deposited in the same sort of curled leaves and rolled petioles of the sierra palm (*Prestoea montana*) that frogs use for daytime retreat sites (Townsend, 1984). Males usually brood their eggs from the time of oviposition until hatching, or even several days beyond (Townsend *et al.*, 1984).

Although *E. coqui* is found at high densities in forested habitat, it will readily use marginal habitats, including human-altered ones. Frogs may be collected by searching areas around houses, gardens, or other human-altered habitats that have sufficient vertical substrates or vegetation on which frogs can perch. Densities in such marginal habitats may be lower, and females may be more difficult to locate. An alternative method is to "seed" an area with small enclosed cavities, such as short pieces of PVC pipe or short sections of bamboo stems, which mimic the natural retreat sites of coquies. Frogs will readily use these for retreats and nest sites if available. Because it requires some amount of time for frogs to discover these new sites and then occupy them, such a technique works best for investigators doing long-term field work or making repeated visits to the field.

Terraria for laboratory maintenance. Eleutherodactylus coqui is an ideal laboratory animal. Individuals of the species can be easily maintained in captivity for several years and will breed in laboratory colonies for up to 1.5 years after collection (Townsend, unpub. data).

Coquíes will live under a variety of conditions, but in our experience, healthy colonies are best maintained in glass aquaria with lids that allow some ventilation. Frogs can be kept in 12–18 gallon aquaria with screen tops that have been partially blocked with plastic wrap or some other transparent waterproof material, the position of which can be adjusted to mediate relative humidity within the tank. Although accustomed to an environment with high relative humidity, coquíes do not tolerate well constant humidity near saturation with little or no air exchange. Periodic wetting and partial drying of laboratory aquaria seem to provide the best conditions for maintaining frogs with maximum vigor and reproductive capacity.

Frogs should not be kept under crowded conditions. A rule of thumb is a minimum of 1.5 gallons of tank capacity per frog. If the object of the research is to encourage breeding, the investigator will want to maximize the number of males and females that can interact while avoiding possible inhibition of calling by males that are too closely spaced. Males maintain a certain minimum nearest neighbor distance in the field, below which they will challenge one another acoustically, or one will stop calling. In such situations, it would be better to keep frogs in larger aquaria or tanks.

Contents of holding tanks may vary, but must include at least two items: one site per frog to be used as a protected daytime retreat site, and a source of water for rehydration. Pieces of 1–1.5 inch diameter PVC pipe or plexiglass tubing, cut into 3–8 inch lengths, serve as excellent retreat sites. Frogs readily adopt these as retreat sites, and males will nest in them as well. If placed in a more or less vertical position in the aquarium, these also serve as elevated perch sites. This species spends a good deal of its active nocturnal period perched above the ground. It is unnecessary to provide standing water for coquíes. Several petri dishes or finger bowls filled with sand, gravel, or a soil mixture that will hold water should be placed in each aquarium. Frogs will sit in these at night and take up water through their ventral abdomen. The bottom of the aquarium may be covered with gravel, sand, or soil to serve as an absorbent medium for urine and a source of water vapor to maintain appropriate humidity within the aquarium. If frogs are to be maintained at fairly low densities, the aquarium may be lined with moss or planted, but such an arrangement makes periodic cleaning difficult. If maintained at higher densities, it is best to cover the bottom with a medium that is more easily cleaned, such as sand or gravel and to add potted plants. Frogs will climb the pots and perch in the potting soil or leaves of the plants. It is best *not* to have standing water in the aquarium bottom. When holding tanks are too wet, either from excessive standing water or stagnant, saturated air, frogs can become edematous, especially in the thighs. This leads to loss of vigor and in some cases, death.

Feeding. Feeding of adult frogs is straightforward. All adults readily eat domestic crickets (*Acheta domesticus*) if the crickets are alive and active. At temperatures between 20–25°C, an individual frog need be fed only 3–4 adult crickets per week. We recommend that crickets be dusted with a mineral or vitamin supplement twice a month to cover possible dietary deficiencies from such a highly restricted diet. Frogs do not feed well on mealworms, and *Drosophila* are generally too small and active to serve as a good food source. Unlike *Gastrotheca*, coquíes cannot be trained to take non-moving food.

Hatchling froglets average 6 mm in size and are extremely difficult to maintain, principally because of difficulty with feeding. *Drosophila* are actually too large, and even wingless forms will hop about too rapidly for the young to catch. A large portion of the diet of these tiny froglets in the wild consists of tiny arthropods including

ants, arachnids, beetles, and probably collembolans, all less than 3 mm in length (Townsend, 1985). We have been marginally successful with feeding very small *Collembola* (springtails), but colonies of these insects can be difficult to maintain at sufficient densities to provide a long-term constant food supply.

Reproductive behavior. Because developmental studies may be concerned with very early development, it is important to know the schedule of events around fertilization in *E. coqui*. This species practices internal fertilization (Townsend *et al.*, 1981). Consequently, eggs are already fertilized and on their way to first cleavage by the time of egg laying (Elinson, 1987b). Fertilization seems to occur 1–3 h prior to egg laying. By observing the mating and courtship behavior of *E. coqui*, the investigator can identify with some precision when fertilization is taking place and can be alert for the appearance of eggs or be able to sacrifice females to obtain eggs before laying.

Nocturnally active, male coquíes give advertisement calls to attract gravid females. Calling starts shortly after dusk and often continues into the early morning. In the field, a gravid female approaches a calling male, usually before midnight, and makes contact with him. The male then leads the female to a prospective nest site. If the female enters and remains in the nest site, the pair remain in contact, with the female beneath the male although she is never clasped by him. After 5.5–7.5 h, they adopt a unique position in which the female, while still beneath the male, places her hind legs on top of his (Townsend and Stewart, 1986a). The adoption of this unique "reverse hind leg clasp" (Fig. 3) is a valuable cue to the investigator that the female will soon ovulate, usually within an hour. Males probably inseminate females soon after adoption of the reverse hind leg clasp as well. Ovulation seems to involve major body spasms, culminating in abdominal contractions by the female. These can continue for over two hours before the first eggs are actually laid. It is during the prelaying period of spasms and contractions that freshly fertilized eggs may be obtained by anesthetizing the female and dissecting the eggs from her ovisac, a common junction of the paired oviducts. Because of the long interim period between the female's entry into the nest site and adoption of the reverse hind leg clasp by the pair, it is possible to anticipate laying with some accuracy. Egg laying usually occurs within 8.5–12 h of the pair initially entering the nest, and within 2–3 h of adoption of the reverse hind leg clasp. Because initial contact between a calling male and a gravid female typically occurs during midevening, egg laying usually occurs conveniently between 7 am and noon the following morning. The investigator just has to check the male retreat sites each morning to see if there will be freshly fertilized eggs that day.

Under ideal laboratory conditions, a female coqui produces a clutch of about 40 eggs every 5–8 weeks. Females only deposit clutches following courtship with calling males; attempts to trigger ovulation by injection of hCG or other gonadotropins have not been successful. It appears that ovulation requires the appropriate sensory signals derived from some aspect of courtship, amplexus, or internal fertilization.

Figure 3. Reverse hind leg clasp during amplexus in *Eleutherodactylus coqui*. This unusual position, with the female's legs on top of the male's legs, is a sign that egg-laying will soon begin. (Drawn from photograph in Townsend and Stewart, 1986a.)

Culture of embryos

The male broods the eggs for about three weeks during which time the eggs develop to hatched froglets (Townsend *et al.*, 1984). The clutch can be removed from the male and his nest site, however, and raised in several ways. A typical culturing method is to place the eggs on absorbent filter paper soaked with spring water in a closed Petri dish. Eggs undergo normal development as long as the filter paper is kept wet, and temperatures are maintained at 21–25°C. Embryos are staged according to Townsend and Stewart (1985).

While development is excellent under these conditions, the embryos are enclosed in their jelly capsules and are not accessible to experimental manipulation. For exper-

imental purposes, embryos can be cultured in water (Lynn and Peadon, 1955) or in 20% Steinberg's solution (Elinson, 1987b). Jellied embryos are submerged in 20% Steinberg's, and the outer and middle jelly layers are removed with watchmaker's forceps. Embryos with intact jelly show retarded development, possibly because of reduced gas exchange. It is initially difficult to remove the fertilization envelope and inner jelly layer, but it becomes easy to do so by Townsend/Stewart stage 7. The embryos continue to develop in 20% Steinberg's until Townsend/Stewart stages 14–15 when they are allowed to crawl onto land. Development of embryos from most clutches occurs normally in 20% Steinberg's, although embryos in some clutches develop edema around Townsend/Stewart stage 7.

Normal development

Eleutherodactylus coqui lends itself to the study of development because of several features. Eggs are large, averaging 4 mm, and are virtually unpigmented for the first two-thirds of development. Many internal features, including the cardiovascular system, nervous system, the development of eyes and ears, and the formation of endolymphatic calcium deposits, are clearly visible in the rather translucent embryo (Townsend and Stewart, 1985). Developmental studies have been conducted on several *Eleutherodactylus* species, including studies by Sampson (1904), Lynn (1942), Gitlin (1944), Lynn and Lutz (1946, 1947), Goin (1947), Jameson (1950), Hughes (1959, 1962), Adamson *et al.* (1960), Chibon (1960), Valett and Jameson (1961), Wake (1978), and Townsend and Stewart (1985). Detailed histological descriptions of organ formation are provided by Lynn (1942), Adamson *et al.* (1960), and Chibon (1962).

Unlike the many normal tables available for *Rana* species, *X. laevis*, and other species with typical embryonic development followed by a free-living larval stage, there are few detailed tables for direct developing anurans. As a first attempt at a general staging scheme to characterize gross external changes in morphology, Townsend and Stewart (1985) proposed a 15-stage scheme that covers development from fertilization to hatching in approximately equal time intervals. This normal table was designed with field studies in mind, so it provides only three stages for the period from fertilization to neurulation. A generalized table for aquatic species (Gosner, 1960) can be used for the preneurula stages. A synopsis of the most distinctive aspects of each of the Townsend/Stewart stages will be mentioned here (Fig. 4).

Stages 1 and 2 cover blastulation and gastrulation, respectively. As with *G. riobambae* and other large-egged amphibians, the blastocoel roof of the *E. coqui* embryo is translucent. Unlike *G. riobambae*, a large, prominent

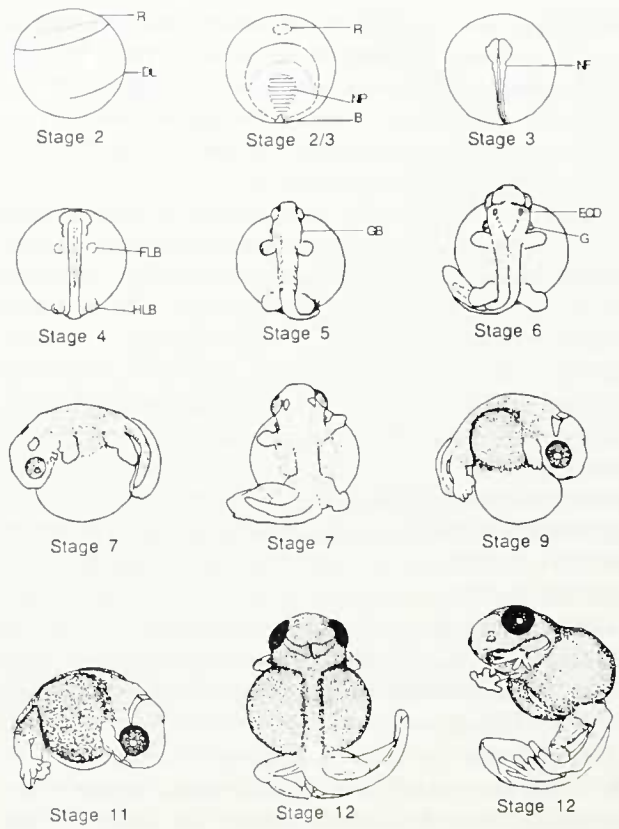

Figure 4. Selected Townsend/Stewart stages of *Eleutherodactylus coqui* development. Gastrulation (Stage 2, lateral view; Gosner Stage 10) is marked by a prominent dorsal lip (DL) of the blastopore, similar to frogs with aquatic development. The embryo, however, is all white with a translucent blastocoel roof (R). A dorsal view of a late gastrula/early neurula (Stage 2/3; Gosner Stage 13) shows the neural plate (NP), an almost closed blastopore (B), and a clear window of uncovered blastocoel roof. Different shades of white allow one to distinguish the neural plate, the rest of the archenteric roof, and the advancing edge of the archenteron. The neurula (Stage 3; Gosner Stage 15) has clear neural folds (NF). Front and hind limb buds (FLB, HLB) form rapidly (Stage 4), and gill buds (GB) become visible (Stage 5). White endolymphatic calcium deposits (ECD) mark Stage 6. The pigmented body wall advances over the yolky cells, the ECD enlarge, and the digits become distinct (Stages 7, 9, 11, 12). The embryo, in its jelly capsule, has a vascularized paddle-shaped tail wrapped around its body to provide a respiratory surface. (Modified from Townsend and Stewart, 1985.)

dorsal lip of the blastopore indicates the start of gastrulation in *E. coqui*. The presence of a neural plate and neural folds indicates the onset of stage 3. These first three stages take 3–4 days in *E. coqui* compared to about 2–3 weeks for the comparable development in *G. riobambae*.

Stage 4 is distinguished by the paired appearance of pimple-like swellings on the surface of the yolk lateral to the neural tube. These are incipient limb buds. Stage 5 is indicated by early limb buds clearly attached to the main trunk and by the initiation of gill buds. Stage 6 is marked by the first appearance of pigment in the eyes and the first evidence of endolymphatic calcium deposits (ECD) as

paired points of pure white material lateral to the junction of midbrain and hindbrain. In stage 7, elbow and knee joints are first evident, and the single pair of gills exhibit their maximum development. Gills are never covered by an operculum in this frog. In stage 8, embryos exhibit the first signs of digits, particularly on hind limbs, and ECD are quadrangular patches with slight anterolateral extensions. From stage 6 to stage 9, the head and trunk of the developing embryo slowly gain pigment. By stage 9, a moderately pigmented body wall surrounds approximately ⅓ of the large yolk supply. Also by stage 9, the single pair of gills has disappeared from external view. Stage 10 is marked by eyes with completely darkened irises while the pupils are still clear. The ECD extend anterolaterally to the rear of the eye but are still separated by a gap medially, and a pigmented body wall surrounds ½–⅔ of the yolk. In stage 11 embryos, toes reach about half of their final length and the pigmented body wall finishes enclosing the yolk reserve. Stage 12 is a longer period, during which the ECD articulate at the embryo's midline and form a broad, shallow U with somewhat broadened base and spread arms. The pigmented body wall completely encloses the yolk, and the pigmentation wall is heavy enough to begin obscuring the ECD at this stage. Stage 13 is marked by full length toes with slight swellings at their tips indicative of incipient toe discs. The first evidence of pigmented eyelids appears, and the initial regression of the unpigmented tail begins. Stage 14 embryos possess toes with full toe discs, eyes with adult coloration (iris golden-brown above, bronze-brown below; pupil black), and clear banding patterns on the hind legs.

Coquí eggs may hatch anytime during stage 15. Embryos at this stage possess full coloration, including any of several distinct pattern morphs exhibited by the species. The remains of the ECD are completely masked by dorsal pigmentation. A bifurcate, black egg tooth at the symphysis of the upper jaw, first evident in stage 12 or 13, is apparently used by the froglet to rupture the egg membrane and hatch.

Development at 25°C requires approximately 17 days (Townsend and Stewart, 1986b). Hatchlings average 6 mm in length, and possess a tail remnant that requires up to two days to resorb after hatching. The large yolk reserve usually lasts for up to a week.

Manipulation of embryos

The normally terrestrial embryos of *Eleutherodactylus* can be cultured in water or in simple salt solutions as described earlier. This permits various experimental manipulations such as surgery or chemical treatments which would otherwise be very difficult.

For instance, Lynn and Peadon (1955) were interested in the role of thyroxine in the development of *E. martinicensis*, a direct developer like *E. coqui*. Thyroxine causes metamorphosis from tadpoles to adults in frogs with aquatic development. Embryos were freed from the jelly and fertilization membrane at Townsend/Stewart stages 6–7, and cultured in tap water with either thyroxine or phenylthiourea, a thyroid inhibitor. Thyroxine caused regression of the tail and degeneration of the pronephros. Limb development, however, was not under thyroid control as it is in metamorphosing amphibians. It is clear that thyroxine and other chemicals given exogeneously can enter these embryos.

Surgery is also possible. Hughes (1962) transplanted limb buds between embryos of *E. martinicensis* to see the interaction between the developing limb and the nervous system. Embryos as early as Townsend/Stewart stage 6 were removed from their jelly capsules and initially cultured in the capsular fluid. A limb bud was cut from one embryo and grafted via a small incision to a second embryo. After the operation, the embryo was allowed to heal for a day in Holtfreter's solution (approximately equivalent to 100% Steinberg's). Thereafter the embryo was cultured in water.

From these limited experiences, it appears easy to experiment on embryos older than Townsend/Stewart stage 6. Whether earlier embryos can be manipulated depends very much on whether they can be removed from the jelly capsule without injury. A second difficulty is that the early embryo may require the structural support provided by the jelly capsule. The use of dishes with an agar base or filled with Percoll (Pharmacia), a high density, low tonicity solution, may permit culturing of early embryos without jelly.

Conclusions

Terrestrial-breeding frogs provide opportunities for analysis by both developmental and evolutionary biologists (Elinson, 1987a, 1990). Obvious comparative and evolutionary questions include:

a. How does large egg size affect early development?

b. How are reproductive adaptations, such as the female's pouch or the male's brooding behaviour, controlled hormonally?

c. What is the cellular and hormonal basis of direct development, an ontogeny without metamorphosis?

d. How did the different reproductive and developmental patterns evolve?

Beyond these questions, embryos from terrestrial-breeding frogs would be ideal for examining certain developmental problems. For instance, the translucent blastocoel roof in both *G. riobambae* and *E. coqui* would allow the migration of internal cells during gastrulation to be followed in intact embryos. The early development

of large limb buds in *E. coqui* would permit the analysis of pattern formation in limbs. Finally, the eggs and embryos themselves present unique questions for cell and developmental biologists. Certainly the formation of oocytes with hundreds of nuclei and the controlled degeneration of them is an intriguing problem for the future.

In this article, we have described how to obtain eggs and embryos for the laboratory investigation of the unusual ontogenies found in these frogs. While these recipes permit the investigator to work in Europe or North America, we would urge that anyone interested in these and other such anurans, travel to their country of origin. This would not only allow the observation of the animals in their native habitats, but would also promote the interaction between scientists from temperate and tropical zones.

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Literature Cited

Adamson, L., R. G. Harrison, and I. Bayley. 1960. The development of the whistling frog *Eleutherodactylus martinicensis* of Barbados. *Proc. Zool. Soc. Lond.* **133:** 453–469.

Auber-Thomay, M., and F. Letellier. 1986. Observations sur le développement de la rainette marsupiale, *Gastrotheca riobambae* (Hylidés). *Rev. Fr. Aquariol.* **13:** 79–86.

Chibon, P. 1960. Développement au laboratoire d'*Eleutherodactylus martinicensis* Tschudi, batracien anoure a développement direct. *Bull. Soc. Zool. Fr.* 85: 412–418.

Chibon, P. 1962. Différenciation sexuelle de *Eleutherodactylus martinicensis* Tschudi, batracien anoure a développement direct. *Bull. Soc. Zool. Fr.* **87:** 509–515.

de Albuja, C. M., M. Campos, and E. M. del Pino. 1983. Role of progesterone on oocyte maturation in the egg-brooding hylid frog *Gastrotheca riobambae* (Fowler). *J. Exp. Zool.* **227:** 271–276.

del Pino, E. M. 1980. Morphology of the pouch and incubatory integument in marsupial frogs (Hylidae). *Copeia* **1980:** 10–17.

del Pino, E. M. 1983. Progesterone induces incubatory changes in the brooding pouch of the frog *Gastrotheca riobambae* (Fowler). *J. Exp. Zool.* **227:** 159–163.

del Pino, E. M. 1989. Modifications of oogenesis and development in marsupial frogs. *Development* **107:** 169–187.

del Pino, E. M. and R. P. Elinson. 1983. A novel development pattern for frogs: gastrulation produces an embryonic disk. *Nature* **306:** 589–591.

del Pino, E. M., and B. Escobar. 1981. Embryonic stages of *Gastrotheca riobambae* (Fowler) during maternal incubation and comparison of development with that of other egg-brooding hylid frogs. *J. Morphol.* **167:** 277–296.

del Pino, E. M., and A. A. Humphries Jr. 1978. Multiple nuclei during early oogenesis in *Flectonotus pygmaeus* and other marsupial frogs. *Biol. Bull.* **154:** 198–212.

del Pino, E. M., M. L. Galarza, C. M. de Albuja, and A. A. Humphries Jr. 1975. The maternal pouch and development in the marsupial frog *Gastrotheca riobambae* (Fowler). *Biol. Bull.* **149:** 480–491.

del Pino, E. M., H. Steinbeisser, A. Hofmann, C. Dreyer, M. Campos, and M. F. Trendelenburg. 1986. Oogenesis in the egg-brooding frog *Gastrotheca riobambae* produces large oocytes with fewer nucleoli and low RNA content in comparison to *Xenopus laevis*. *Differentiation* **32:** 24–33.

Drewry, G. E. and K. L. Jones. 1976. A new ovoviviparous frog, *Eleutherodactylus jasperi* (Amphibia, Anura, Leptodactylidae), from Puerto Rico. *J. Herpetol.* **10:** 161–165.

Duellman, W. E. 1977. Liste der rezenten Amphibien und Reptilien. Hylidae, Centrolenidae, Pseudidae. In *Das Tierreich*, M. Mertens, W. Hennig, and H. Wermuth, eds. Walter de Gruyter & Co., Berlin. 95: 230 pp.

Duellman, W. E. 1980. A new species of marsupial frog (Hylidae: *Gastrotheca*) from Venezuela. *Univ. Mich. Mus. Zool. Occas. Pap.* **690:** 1–7.

Duellman, W. E. 1983. A new species of marsupial frog (Hylidae: *Gastrotheca*) from Colombia and Ecuador. *Copeia* **1983:** 868–874.

Duellman, W. E. 1984. Taxonomy of Brazilian hylid frogs of the genus *Gastrotheca*. *J. Herpetol.* **18:** 302–312.

Duellman, W. E., and P. Gray. 1983. Developmental biology and systematics of the egg-brooding hylid frogs, genera *Flectonotus* and *Fritziana*. *Herpetologica* **39:** 333–359.

Duellman, W. E., and D. M. Hillis. 1987. Marsupial frogs (Anura: Hylidae: *Gastrotheca*) of the Ecuadorian Andes: resolution of taxonomic problems and phylogenetic relationships. *Herpetologica* **43:** 141–173.

Duellman, W. E., and M. S. Hoogmoed. 1984. The taxonomy and phylogenetic relationships of the hylid frog genus *Stefania*. *Univ. Kans. Misc. Publ.* **75:** 1–39.

Duellman, W. E., and S. J. Maness. 1980. The reproductive behavior of some hylid marsupial frogs. *J. Herpetol.* **14:** 213–222.

Duellman, W. E., and R. A. Pyles. 1980. A new marsupial frog (Hylidae: *Gastrotheca*) from the Andes of Ecuador. *Occas. Pap. Mus. Nat. Hist. Univ. Kans.* **84:** 1–13.

Duellman, W. E., and L. Trueb. 1986. *Biology of Amphibians*. McGraw-Hill Book Co., New York.

Elinson, R. P. 1987a. Change in developmental patterns: embryos of amphibians with large eggs. Pp. 1–21 in *Development as an Evolutionary Process*, R. A. Raff and E. C. Raff, eds. Alan R. Liss, Inc., New York.

Elinson, R. P. 1987b. Fertilization and aqueous development of the Puerto Rican terrestrial-breeding frog, *Eleutherodactylus coqui*. *J. Morphol.* **193:** 217–224.

Elinson, R. P. 1990. Direct development in frogs: wiping the recapitulationist slate clean. *Semin. Dev. Biol.* (in press).

Elinson, R. P. and E. M. del Pino. 1985. Cleavage and gastrulation in the egg-brooding, marsupial frog, *Gastrotheca riobambae*. *J. Embryol. Exp. Morphol.* **90:** 223–232.

Gitlin, D. 1944. The development of *Eleutherodactylus portoricensis*. *Copeia* **1944:** 91–98.

Goin, C. J. 1947. Studies on the life history of *Eleutherodactylus ricordii planirostris* (Cope) in Florida with special reference to the local distribution of an allelomorphic color pattern. *Univ. Fl. Stud. Biol. Sci. Ser.* **4:** 1–66.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologia* **16:** 183–190.

Gurdon, J. B. 1968. Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. *J. Embryol. Exp. Morphol.* **20:** 401–414.

Hoogmoed, M. S. 1967. Mating and early development of *Gastrotheca marsupiata* (Duméril and Bibron) in captivity (Hylidae, anura, amphibia). *Br. J. Herpetol.* **4:** 1–7.

Hughes, A. 1959. Studies in embryonic and larval development in Amphibia. I. The embryology of *Eleutherodactylus ricordii*, with special reference to the spinal cord. *J. Embryol. Exp. Morphol.* **7:** 22–38.

Hughes, A. 1962. An experimental study on the relationships between limb and spinal cord in the embryo of *Eleutherodactylus martinicensis*. *J. Embryol. Exp. Morphol.* **10:** 575–601.

Jameson, D. L. 1950. The development of *Eleutherodactylus latrans*. *Copeia* **1950:** 44–46.

Jones, R. E., A. M. Gerrard, and J. J. Roth. 1973. Estrogen and brood pouch formation in the marsupial frog *Gastrotheca riobambae*. *J. Exp. Zool.* **184:** 177–184.

Kageyama, T. 1980. Cellular basis of epiboly of the enveloping layer in the embryo of medaka, *Oryzias latipes*. I. Cell architecture revealed by silver staining method. *Dev. Growth Differ.* **22:** 659–668.

Lamotte, M. and J. Lescure. 1977. Tendances adaptives a l'affranchissement du milieu aquatique chez les amphibiens anoures. *Terre Vie* **31:** 225–311.

Laurent, R. F., E. O. Lavilla, and E. M. Terán. 1986. Contribución al conocimiento del genero *Gastrotheca* Fitzinger (Amphibia: Anura: Hylidae) en Argentina. *Acta Zool. Lilloana* **38:** 171–221.

Lynn, W. G. 1942. The embryology of *Eleutherodactylus nubicola*, an anuran which has no tadpole stage. *Contrib. Embryol. Carnegie Institute Wash.* **190:** 29–62.

Lynn, W. G., and B. Lutz. 1946. The development of *Eleutherodactylus guentheri* Stdnr. 1846 (Salientia). *Bol. Mus. Nac. Brasil (Nova Ser.)* **71:** 1–46.

Lynn, W. G., and B. Lutz. 1947. The development of *Eleutherodactylus nasutus* Lutz. *Bol. Mus. Nac. Brasil (Nova Ser.)* **79:** 1–30.

Lynn, W. G. and A. M. Peadon. 1955. The role of the thyroid gland in direct development in the anuran, *Eleutherodactylus martinicensis*. *Growth* **19:** 263–286.

Maegregor, H. C., and E. M. del Pino. 1982. Ribosomal gene amplification in multinucleate oocytes of the egg brooding frog hylid *Flectonotus pygmaeus*. *Chromosoma (Berl.)* **85:** 475–488.

Noble, G. K. 1927. The value of life history data in the study of the evolution of the amphibia. *Ann. N. Y. Acad. Sci.* **30:** 31–128.

Rivero, J. A. 1978. *Los anfibios y reptiles de Puerto Rico*, Editorial Universitaria, Universidad de Puerto Rico, San Juan, Puerto Rico.

Rugh, R. 1965. *Experimental Embryology*. Third Edition. Burgess Publ. Co., Minneapolis, Minnesota.

Sampson, L. V. 1904. A contribution to the embryology of *Hylodes martinicensis*. *Am. J. Anat.* **3:** 473–504.

Stewart, M. M. 1985. Arboreal habitat use and parachuting by a subtropical forest frog. *J. Herpetol.* **19:** 391–401.

Townsend, D. S. 1984. The adaptive significance of male parental care in a neotropical frog. Ph.D. dissertation, State University of New York, Albany.

Townsend, D. S., and M. M. Stewart. 1985. Direct development in *Eleutherodactylus coqui* (Anura: Leptodactylidae): a staging table. *Copeia 1985*: 423–436.

Townsend, D. S., and M. M. Stewart. 1986a. Courtship and mating behavior of a Puerto Rican frog, *Eleutherodactylus coqui*. *Herpetologica* **42:** 165–170.

Townsend, D. S., and M. M. Stewart. 1986b. The effect of temperature on direct development in a terrestrial-breeding, neotropical frog. *Copeia* **1986:** 520–523.

Townsend, D. S., M. M. Stewart, F. H. Pough, and P. F. Brussard. 1981. Internal fertilization in an oviparous frog. *Science* **212:** 469–471.

Townsend, D. S., M. M. Stewart, and F. H. Pough. 1984. Male parental care and its adaptive significance in a neotropical frog. *Anim. Behav.* **32:** 421–431.

Townsend, K. 1985. Ontogenetic changes in resource use by the Puerto Rican frog *Eleutherodactylus coqui*. M.Sc. Thesis, State University of New York, Albany.

Trueb, L. 1974. Systematic relationships of neotropical horned frogs, genus *Hemiphractus* (Anura, Hylidae). *Occas. Pap. Mus. Nat. Hist. Univ. Kans.* **29:** 1–60.

Trueb, L. and Duellman, W. E. 1978. An extraordinary new casque-headed marsupial frog (Hylidae: *Gastrotheca*). *Copeia* **1978:** 498–503.

Valett, B. B., and D. L. Jameson. 1961. The embryology of *Eleutherodactylus augusti latrans*. *Copeia* **1961:** 103–109.

Wake, M. H. 1978. The reproductive biology of *Eleutherodactylus jasperi* (Amphibia, Anura, Leptodactylidae), with comments on the evolution of live-bearing systems. *J. Herpetol.* **12:** 121–133.

Wolf, D. P., and J. L. Hedrick. 1971. A molecular approach to fertilization. II. Viability and artificial fertilization of *Xenopus laevis* gametes. *Dev. Biol.* **25:** 348–359.

Zimmermann, E. 1983. *Das Züchten von Terrarientieren: Pflege, Verhalten, Fortpflanzung*. Kosmos Bücher, Franckh'sche Verlagshandlung, W. Keller & Co., Stuttgart.